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(54) Title: POLYPHENOL OXIDASE GENES FROM LETTUCE AND BANANA

(57) Abstract

The present invention provides methods for preparing nucleic acids encoding polyphenol oxidase (PPO), fragments and derivatives thereof. The present invention also provides nucleic acids encoding banana or lettuce PPO, or antisense to banana or lettuce PPO, fragments and derivatives thereof. Vectors including such nucleic acids, methods of using such nucleic acids and transgenic plants are also provided.

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POLYPHENOL OXIDASE GENES FROM LETTUCE AND BANANA

The present invention relates to the isolation of genes encoding polyphenol oxidase (PPO) from plants.

Browning of plant tissues often occurs following injury or damage and this generally results in spoilage of fruit and vegetables. Undesirable browning also occurs during processing of plant materials to produce food or other products. Steps are taken during transport, storage, and processing to prevent these browning reactions. Often this involves the use of chemicals such as sulphur dioxide but the use of these substances is likely to be restricted in the future due to concerns about their safety and consumer acceptance. For example, the US Food and Drug Administration banned the use of sulphite for most fresh fruit and vegetables in 1986. The production of fruit and vegetable varieties with an inherently low susceptibility to brown would remove the need for these chemical treatments.

It will be understood that browning in plants is predominantly catalysed by the enzyme PPO. PPO is localised in the plastids of plant cells whereas the phenolic substrates of the enzyme are stored in the plant cell vacuole. This compartmentation prevents the browning reaction from occurring unless the plant cells are damaged and the enzyme and its substrates are mixed.

The prior art includes International Application PCT/AU92/00356 to the present applicant which describes the cloning of PPO genes from grapevine, broad bean leaf, apple fruit and potato tuber. This application recognises that PPO levels in plants may be manipulated by increasing or decreasing expression of PPO gene. The application also identifies two conserved copper binding sites in PPO genes, designated CuA and CuB. However, the method described in PCT/AU92/00356 which was used to clone the PPO genes from apple and potato involved the use of an oligo dT reverse primer for polymerase chain reaction (PCR). Whilst the method is acceptable, in some tissues, it does not give rise to a strong band of the predicted size or else it gives rise to many additional products making it difficult to resolve the PPO fragment.

Accordingly, it is an object of the present invention to overcome or at least alleviate one or more of the difficulties related to the prior art.

In a first aspect of the present invention there is provided a method for preparing nucleic acid encoding PPO, fragments and derivatives thereof, which method includes

providing

a source of a polypeptide having PPO activity,

a first primer having a sequence corresponding to a first conserved region of PPO in sense orientation, and

a second primer having a sequence corresponding to a second conserved region of PPO in antisense orientation;

isolating RNA from the source of polypeptide having PPO activity;

treating the RNA to construct copy DNA (cDNA) therefrom; and

amplifying the cDNA so formed using the first and second primers.

Applicant has found that the method of the present invention, which involves the use of a second primer based on PPO, means that there is less likelihood that other (non-PPO) genes are amplified. Furthermore, the method of the present invention dramatically increases the amount of genuine product formed in most cases. Moreover, the added specificity provided by the second PPO-based primer makes it possible to clone PPO more readily from certain plants in which it was difficult to obtain a clone using one primer and oligo-dT. For example, with lettuce cDNA the applicant saw only a faint smear of a range of products with GEN3/GEN8 and oligo-dT but strong bands of the predicted size with GEN3/GEN8 and REV1.

The terms "nucleic acid encoding banana/lettuce PPO" and "banana/lettuce PPO gene" as used herein should be understood to refer to a banana/lettuce PPO gene or a sequence substantially homologous therewith. For example, these terms include sequences which differ from the specific sequences given in the Examples hereto but which, because of the degeneracy of the genetic code, encode the same protein. Applicants have found that there are families of PPO genes in most plants. Thus, there are likely to be other PPO genes in lettuce and banana, in addition to those which have been isolated. These could be cloned using the methods of the present invention. Thus, the terms "nucleic acid encoding banana/lettuce PPO" and "banana/lettuce PPO

gene" should be understood to include banana/lettuce PPO genes other than those specific genes that have been isolated. The terms may also include presequences such as chloroplast transit sequence as well as sequences encoding mature PPO protein.

The term "derivative" as used herein includes nucleic acids that have been chemically or otherwise modified, for example mutated, or labelled, or nucleic acids incorporating a catalytic cleavage site.

The term "fragment" includes functionally active fragments of a PPO gene which encode a polypeptide or peptide having PPO activity.

The source of polypeptide having PPO activity is preferably a source of polypeptide having banana or lettuce PPO activity. The source of polypeptide having banana PPO activity may be banana fruit, preferably young banana fruit, more preferably the flesh of young banana fruit. The source of polypeptide having lettuce PPO activity may be lettuce leaves, preferably young lettuce leaves.

The RNA may be isolated by any suitable method including extraction for example with a detergent such as CTAB, use of an oligo-dT spun column as described in PCT/AU92/00356 the entire disclosure of which is incorporated herein by reference, or use of a commercially available kit such as the PolyATtract 1000 system from Promega Corporation.

The step of treating the RNA to construct cDNA according to this aspect of the present invention may include

treating the RNA with reverse transcriptase and an adapter primer to form cDNA.

The adapter primer may be an oligonucleotide adapter primer including the following sequence or part thereof:

5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3'

The first primer has a sequence corresponding to a first conserved region of PPO. Preferably the first primer has a sequence corresponding to at least a portion of or in close proximity to a first copper binding site of PPO. The second primer has a sequence corresponding to a second conserved region of PPO. Preferably the second primer has a sequence corresponding to at least a portion

of or in close proximity to a second copper binding site of PPO. More preferably the first primer has a sequence corresponding to at least a portion of or in close proximity to one of the CuA or CuB binding sites of PPO, and the second primer has a sequence corresponding to at least a portion of or in close proximity to the other of the CuA or CuB binding sites of PPO.

The first and second primers may be degenerate. The first primer may include one of the following sequences or part thereof:

5'-GCGAATTCTT[TC][TC]TICCITT[TC]CA[TC][AC]G-3'

5'-GCGAATTCGATCCIACITT[TC]GC[GT]TTICC-3'.

The second primer may include the following sequence or part thereof

5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3'.

The cDNA may be amplified using the polymerase chain reaction (PCR).

Those skilled in the art will appreciate that if the Cu binding sites are internal, the nucleic acid isolated will be a fragment of the PPO gene lacking 3' and 5' termini. However, it is possible to determine the complete nucleic acid sequence of the PPO gene and to prepare or isolate nucleic acid encoding such PPO or antisense to such PPO.

Accordingly, in a further aspect of the present invention there is provided a method for preparing nucleic acid encoding the 3' end of PPO, which method includes

providing

a source of polypeptide having PPO activity

a primer in sense orientation; and

an adapter primer;

isolating RNA from the source of polypeptide having PPO activity;

treating the RNA to construct cDNA therefrom; and

amplifying the cDNA so formed using the primers.

In a further aspect of the present invention there is provided a method for preparing nucleic acid encoding the 5' end of PPO, which method includes

providing

a source of polypeptide having PPO activity,

an anchor,

primers in antisense orientation; and

an anchor primer;

isolating RNA from the source of polypeptide having PPO activity;

treating the RNA to construct cDNA therefrom;

attaching the anchor to the 5' end of the cDNA so formed; and

amplifying the cDNA using the primers.

The source of polypeptide having PPO activity is preferably a source of polypeptide having banana or lettuce PPO activity. The source of polypeptide having banana PPO activity may be banana fruit, preferably young banana fruit, more preferably the flesh of young banana fruit. The source of polypeptide having lettuce PPO activity may be lettuce leaves, preferably young lettuce leaves.

The RNA may be isolated by any suitable method including extraction for example with a detergent such as CTAB, use of an oligo-dT spun column as described in PCT/AU92/00356 the entire disclosure of which is incorporated herein by reference, or use of a commercially available kit such as the PolyATtract 1000 system from Promega Corporation.

The step of treating the RNA to construct cDNA according to this aspect of the present invention may include

treating the RNA with reverse transcriptase and an adapter primer to form cDNA.

The adapter primer may be an oligonucleotide adapter primer including the following sequence or part thereof:

5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3'

The primer in sense orientation may be a lettuce PPO specific primer. The primer in sense orientation may include the following sequence or part thereof:

5'-CGCTGGGTGGGTAATTCTAGGATG-3'.

The primer in sense orientation may be a banana PPO specific primer. The primer in sense orientation may include the following sequence or part thereof:

5'-AGTCATCCACAATGCGGCGCACATG-3'.

The adapter primer may include the following sequence or part thereof:

5'-GACTCGAGTCGACATCG-3'.

The primers in antisense orientation may be lettuce PPO specific primers. The primers in antisense orientation may include the following sequences or part thereof:

5'-TGCTGTTCTGTTCGAACATGGCAG-3'

5'-TATACAAGTGGCACCAGTGTCTGC-3'.

The primers in antisense orientation may be banana PPO specific primers. The primers in antisense orientation may include the following sequences or part thereof:

5'-CCGCATTGTGGATGACTTCCATCTG-3'

5'-CCAGAATGGGATGGTGAAGGTGTCG-3'.

The anchor may be of any suitable type. The anchor may be attached by ligation for example using T4 RNA ligase. The anchor primer should be capable of hybridizing with the anchor.

The cDNA may be amplified using PCR.

Those skilled in the art will appreciate that using the methods of the present invention it is possible to determine the complete nucleic acid sequence of the PPO gene of interest and to prepare or isolate nucleic acid encoding such PPO or antisense to such PPO.

In a further aspect of the present invention, there is provided a nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof. Preferably the nucleic acid has the sequence shown in Fig. 2 or Fig. 3, fragments and derivatives thereof, and substantially homologous sequences.

In a further aspect of the present invention, there is provided a nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof. Preferably the nucleic acid has the sequence shown in Fig. 1, fragments and derivatives thereof, and substantially homologous sequences.

The nucleic acid may be prepared by a method as hereinbefore described.

The nucleic acid may be modified, for example by inclusion of a catalytic cleavage site.

In a further aspect of the present invention there is provided a method for preparing a recombinant vector including a nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof, which method includes

providing

nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof; and

a vector; and

reacting the nucleic acid and the vector to deploy the nucleic acid within the vector.

In a further aspect of the present invention there is provided a method for preparing a recombinant vector including a nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof, which method includes

providing

nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof; and

a vector; and

reacting the nucleic acid and the vector to deploy the nucleic acid within the vector.

The nucleic acid may be prepared by a method as hereinbefore described.

The nucleic acid may be modified, for example by inclusion of a catalytic cleavage site.

The vector may be a plasmid expression vector. For example Bluescript SK^+ has been found to be suitable. Alternatively, the vector may be a binary vector. The recombinant vector may contain a promoter, preferably a constitutive promoter upstream of the nucleic acid.

The cloning step may take any suitable form. A preferred form may include

fractionating the cDNA, for example on a column or a gel;

isolating a fragment of the expected size, for example from the column or gel; and

ligating said fragment into a suitable restriction enzyme site of the vector, for example the EcoRV site of a Bluescript SK$^+$ vector.

In order to test the clones so formed, a suitable microorganism may be transformed with the vector, the microorganism cultured and the polypeptide encoded therein expressed. The microorganism may be a strain of Escherichia coli, for example E.coli DH5 has been found to be suitable. Alternatively, appropriate vectors may be used to transform plants.

In a further aspect of the present invention there is provided a recombinant vector including a nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof, which vector is capable of being replicated, transcribed and translated in a unicellular organism or alternatively in a plant.

In a further aspect of the present invention there is provided a recombinant vector including a nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof, which vector is capable of being replicated, transcribed and translated in a unicellular organism or alternatively in a plant.

The nucleic acid may be prepared by a method as hereinbefore described.

The nucleic acid may be modified, for example by inclusion of a catalytic cleavage site.

The vector may be a plasmid expression vector. For example Bluescript SK$^+$ has been found to be suitable. Alternatively, the vector may be a binary vector. The recombinant vector may contain a promoter, preferably a constitutive promoter upstream of the nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof.

The microorganism may be a strain of Escherichia coli, for example E.coli DH5 has been found to be suitable.

In a further aspect of the present invention there is provided a method of decreasing the level of PPO activity in a plant tissue, which method includes

providing

a nucleic acid encoding banana PPO, a modified nucleic acid encoding banana PPO, or a nucleic acid antisense to banana PPO, fragments and derivatives thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

In a further aspect of the present invention there is provided a method of decreasing the level of PPO activity in a plant tissue, which method includes

providing

a nucleic acid encoding lettuce PPO, a modified nucleic acid encoding lettuce PPO, or a nucleic acid antisense to lettuce PPO, fragments and derivatives thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

PPO activity may be decreased by the use of sense constructs (cosuppression). Alternatively the nucleic acid may include a sequence encoding antisense mRNA to banana or lettuce PPO or a functionally active fragment thereof. Alternatively the nucleic acid may encode banana or lettuce PPO or a functionally active fragment thereof and incorporate a catalytic cleavage site (ribozyme). The nucleic acid may be included in a recombinant vector as hereinbefore described. In a preferred aspect, the nucleic acid may be included in a binary vector. In a further preferred aspect, the introduction of a binary vector into the plant may be by infection of the plant with an Agrobacterium containing the binary vector or by bombardment with nucleic acid coated microprojectiles. Methods for transforming banana with Agrobacterium are known to those skilled in the art and are described in, for example, May et al., Bio/technology (1995) 13:486-492, the entire disclosure of which is incorporated herein by reference. Methods for transforming banana by bombardment with DNA coated microprojectiles are known to those skilled in the art and are described in, for example, Sagi et al., Bio/technology (1995) 13:481-485, the entire disclosure of which is incorporated herein by reference. Methods for transforming lettuce using Agrobacterium are known to those skilled in the art and are described in, for example, Michelmore et al., Plant Cell Reports (1987) 6:439-442, and Curtis et al., Journal of Experimental Botany (1994)

45:1141-1149.

In a further aspect of the present invention there is provided a method of increasing the level of PPO activity in a plant tissue, which method includes providing

a nucleic acid encoding banana PPO or a fragment thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

In a further aspect of the present invention there is provided a method of increasing the level of PPO activity in a plant tissue, which method includes providing

a nucleic acid encoding lettuce PPO or a fragment thereof; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic plant.

The nucleic acid may be included in a recombinant vector as hereinbefore described. In a preferred aspect, the nucleic acid may be included in a binary vector. In a further preferred aspect, the introduction of the binary vector into the plant may be by infection of the plant with an Agrobacterium containing the binary vector or by bombardment with nucleic acid coated microprojectiles.

The plant may be of any suitable type. However the method is particularly applicable to banana or lettuce.

In a further aspect of the present invention there is provided a transgenic plant, which plant contains nucleic acid capable of modifying expression of the normal banana PPO gene.

The plant may be of any suitable type. Preferably, the plant is banana.

In a further aspect of the present invention there is provided a transgenic plant, which plant contains nucleic acid capable of modifying expression of the normal lettuce PPO gene.

The plant may be of any suitable type. Preferably, the plant is lettuce.

The nucleic acid may be as hereinbefore described.

In a still further aspect of the present invention there is provided a plant vaccine including nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof.

In a still further aspect of the present invention there is provided a plant vaccine including nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof.

The present invention will now be more fully described with reference to the accompanying Examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the Figures:

FIGURE 1: The composite LPO1 cDNA nucleotide sequence and derived protein sequence encoding both the putative chloroplast transit sequence and the mature lettuce PPO protein.

FIGURE 2: The BANPPO1 cDNA nucleotide sequence and derived protein sequence encoding both the putative chloroplast transit sequence and the mature banana PPO protein.

FIGURE 3: The BANPPO11 cDNA nucleotide sequence and derived protein sequence encoding part of a banana PPO protein.

EXAMPLE 1

Cloning Lettuce PPO Genes

Messenger RNA (mRNA) was isolated directly from young leaves of lettuce using the PolyATtract 1000 system from Promega Corporation. First strand cDNA was synthesised with reverse transcriptase using a Timesaver cDNA Synthesis Kit (Pharmacia Biotech) utilising an oligo-dT primer adapter as described in Frohman, MA (1990) in "PCR Protocols : A Guide to Methods and Applications" (MA Innis, DH Gelfrand, JJ Sninsky and TJ White, eds) Academic Press, New York pp 28-38, the entire disclosure of which is incorporated herein by reference:

B26 : (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3').

Oligonucleotide primers were designed based on known plant PPO DNA sequences in the conserved regions of the gene which encode the copper binding sites, CuA and CuB as described in Dry, IB and Robinson, SP (1994) "Molecular cloning and characterisation of grape berry polyphenol oxidase", Plant Molecular Biology 26 : 495-502, the entire disclosure of which is incorporated herein by reference. Two forward primers designed around the CuA site (GEN3 and GEN8) and one reverse primer designed around the CuB site (REV1) were synthesised:

GEN3 : (5'-GCGAATTCTT[TC][TC]TICCITT[TC]CA[TC][AC]G-3')

GEN8 : (5'-GCGAATTCGATCCIACITT[TC]GC[GT]TTICC-3')

REV1 : (5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3')

Although the primers are in the region of the Cu binding sites, one of them (GEN8) is just outside of what is traditionally accepted to be a Cu binding site of the enzyme.

The first strand cDNA was amplified by the polymerase chain reaction (PCR) essentially according to the method of Frohman using GEN3 and REV1 or GEN8 and REV1 primers, each at a final concentration of 1μM (Dry et al.). Amplification involved an initial program of 2 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 2 min, a slow ramp to 72°C over 2 min and elongation at 72°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min. A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products and the remainder was purified and concentrated using PCR Wizard Prep columns (Promega Corporation).

The purified DNA was cloned into Eco RV-cut Bluescript SK^+ vector (Stratagene) which had been T-tailed with Taq Polymerase and the ligated DNA was introduced into E.coli DH5α by electroporation. Recombinant clones which had an insert of the predicted size were selected and their DNA sequence was determined by automated sequencing. Three putative lettuce PPO clones (LPO316, LPO812 and LPO813) were identified based on their homology to known plant PPO genes.

Using this sequence information a specific forward primer (LET3P) and two reverse primers (LET5P1 and LET5P2) were synthesised:

LET3P : (5'-CGCTGGGTGGGTAATTCTAGGATG'3-)

LET5P1 : (5'-TGCTGTTCTGTTCGAACATGGCAG-3')

LET5P2 : (5'-TATACAAGTGGCACCAGTGTCTGC-3')

To obtain the 3'-end of the lettuce PPO gene, the first strand cDNA described above was amplified by the same PCR procedure using 1μM LET3P primer and 100 nM adapter primer:

B25 : (5'-GACTCGAGTCGACATCG-3').

The amplified cDNA was purified as described above and run on a 2% Nusieve GTG (FMC Bioproducts) agarose gel. A 1000bp fragment was excised from the gel and the DNA was cloned into T-tailed, Eco RV-cut Bluescript SK^+ to yield the 3'- end clones LPO9 and LPO10, which were sequenced.

The 5'-end of the lettuce PPO gene was cloned by a modification of the 5'-RACE procedure originally described by Frohman using a 5'-AmpliFINDER RACE kit (Clontech Laboratories). First strand cDNA was synthesised from mRNA with reverse transcriptase using the LET5P2 primer and an AmpliFINDER anchor was ligated onto the 5'-end of the cDNA. The cDNA was amplified by PCR with LET5P1 primer and the AmpliFINDER anchor primer. The amplified cDNA was purified as described above and run on a 2% Nusieve GTG (FMC Bioproducts) agarose gel. An 850bp fragment was excised from the gel and the DNA was cloned into T-tailed Eco RV-cut Bluescript SK^+ to give the 5'-end clones LPO4, LPO5, LPO6, and LPO7, which were sequenced.

The 5'- and 3'-clones were found to have the predicted overlapping sequences with the original clone and the complete sequence of lettuce PPO (LPO1) was derived by combining the sequences from the various clones (Figure 1).

EXAMPLE 2

Cloning Banana PPO Gen s

Total RNA was isolated from young banana fruit. Fruit tissue (3g) was frozen and ground to a fine powder in liquid nitrogen with a coffee grinder then added to 20 ml of extraction buffer (2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinyl pyrolidone, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2 M NaCl, 0.05% spermidine, 2% β-mercaptoethanol) at 65°C. The extract was mixed with 20 ml of chloroform / IAA then centrifuged for 20 minutes at 5,000 RPM and the aqueous phase was re-extracted with chloroform / IAA. The aqueous phase was filtered through Miracloth and 0.25 volumes of 10 M LiCl were added then the sample was incubated overnight at 4°C before centrifuging for 20 minutes at 8,000 RPM. The supernatant was removed and the pellet was resuspended in 0.5 ml of 1 M NaCl, 0.5% SDS, 10 mM Tris, pH 8.0, 1 mM EDTA. The RNA was extracted once with an equal volume of chloroform / IAA and 2 volumes of ethanol was added. After incubation for 40 mins at -70°C the solution was centrifuged for 15 minutes at 10,000 RPM . The supernatant was removed and the pellet was rinsed with 80% ethanol, drained, and dried. The pellet was resuspended in 50 µl of sterile water.

First strand cDNA was synthesised from 10 µg total RNA with reverse transcriptase as described in Dry, I.B. and Robinson, S.P. (1994) "Molecular cloning and characterisation of grape berry polyphenol oxidase", Plant Molecular Biology 26 : 495-502, the entire disclosure of which is incorporated herein by reference, utilising an oligo-dT primer adapter (Frohman, M.A. (1990) in "PCR Protocols : A Guide to Methods and Applications" (M.A. Innis, D.H. Gelfrand, J.J. Sninsky and T.J. White, eds.) Academic Press, New York pp 28-38, the entire disclosure of which is incorporated herein by reference) :

B26 : (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3')

Oligonucleotide primers were designed based on known plant PPO DNA sequences in the conserved regions of the gene which encode the copper binding sites, CuA and CuB (Dry et al.). A forward primer designed around the CuA site (GEN3) and a reverse primer designed around the CuB site (REV1) were synthesised :

GEN3 : (5'-GCGAATTCTT[TC][TC]TICCITT[TC]CA[TC][AC]G-3')

REV1 : (5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3')

The first strand reaction was amplified by the polymerase chain reaction (PCR) essentially according to the method of Frohman using GEN3 and REV1 primers, each at a final concentration of 1 μM (Dry et al.). Amplification involved an initial program of 2 cycles of denaturation at 94° C for 1 min, annealing at 37° C for 2 min, a slow ramp to 72° C over 2 min and elongation at 72° C for 3 min, followed by 25 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 1 min, and elongation at 72° C for 3 min. A sample of the amplified DNA was run on an agarose gel and stained with ethidium bromide to determine the size of the PCR products and the remainder was purified and concentrated using PCR Wizard Prep columns (Promega Corporation).

The purified DNA was cloned into Eco RV-cut Bluescript SK^+ vector (Stratagene) which had been T-tailed with Taq Polymerase and the ligated DNA was introduced into E. coli DH5α by electroporation. Recombinant clones which had an insert of the predicted size were selected and their DNA sequence was determined by automated sequencing. A putative banana PPO clone (BPO3) was identified based on its homology to known plant PPO genes.

Using this sequence information a specific forward primer (BAN1) and two specific reverse primers (BAN2R and BAN3R) were synthesised:

BAN 1 : (5'-AGTCATCCACAATGCGGCGCACATG-3')

BAN2R : (5'-CCGCATTGTGGATGACTTCCATCTG-3')

BAN3R : (5'-CCAGAATGGGATGGTGAAGGTGTCG-3')

To obtain the 3'-end of this banana PPO gene, the first strand cDNA described above was amplified by the same PCR procedure using 1μM BAN1 primer and 100nM adapter primer:

B25 : (5'-GACTCGAGTCGACATCG-3')

The DNA was amplified using 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min. The amplified DNA was purified using a QIAquick Spin PCR Purification Kit (QIAGEN) and run on a 2% Nusieve GTG (FMC Bioproducts) agarose gel. A 1000bp fragment was excised from the gel and the DNA was cloned into T-tailed Eco RV-cut Bluescript

SK+ to yield the 3'-end clone BPO17, which was sequenced and shown to encode the 3'-end of BPO3.

The 5'-end of BPO3 was cloned by a modification of the 5'-RACE procedure originally described by Frohmann. First strand cDNA was synthesised from banana fruit RNA as described above but utilising the banana PPO specific primer BAN2R. The DNA was tailed with Terminal transferase as described in Frohmann and amplified by PCR with BAN3R and B26 primers, each at a final concentration of 1μM. The DNA was amplified using 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min. The amplified DNA was run on a 1.8% Nusieve GTG (FMC Bioproducts) agarose gel and a 700bp fragment was excised from the gel. The DNA was extracted with a QIAquick Gel Extraction Kit and cloned into T-tailed Eco RV-cut Bluescript SK+ to yield the 5'-end clone BPO26 which was sequenced and shown to encode the 5'-end of BPO3.

The overlapping clones BPO3, BPO17 and BPO26 were fully sequenced in both directions and the sequence of this banana PPO gene (BANPPO1) was derived by combining the sequences (Figure 2).

In the course of these experiments a number of clones were obtained from the banana fruit cDNA by PCR amplification using the B25 primer with one of the degenerate primers based on conserved sequences in other plant PPO genes:

GEN7 : (5'-GCGAATTCAA[TC]GTIGA[TC][AC]GIATGTGG-3')

using the methods described above. Most of these clones were identical to BANPPO1 but one clone, designated BANPPO11, was found to be distinctly different and its sequence is shown in Figure 3.

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

CLAIMS:

1. A method for preparing nucleic acid encoding PPO, fragments and derivatives thereof, which method includes

providing

a source of a polypeptide having PPO activity,

a first primer having a sequence corresponding to a first conserved region of PPO in sense orientation, and

a second primer having a sequence corresponding to a second conserved region of PPO in antisense orientation;

isolating RNA from the source of polypeptide having PPO activity;

treating the RNA to construct copy DNA (cDNA) therefrom; and

amplifying the cDNA so formed using the first and second primers.

2. A method according to claim 1 wherein the first primer has a sequence corresponding to at least a portion of or in close proximity to a first copper (Cu) binding site of PPO and the second primer has a sequence corresponding to at least a portion of or in close proximity to a second Cu binding site of PPO.

3. A method according to claim 2 wherein the nucleic acid encodes banana or lettuce PPO and the source of polypeptide having PPO activity is a source of polypeptide having banana or lettuce PPO activity, respectively.

4. A method according to claim 3 wherein the first primer includes one of the following sequences or part thereof:

5'-GCGAATTCTT[TC][TC]TICCITT[TC]CA[TC][AC]G-3'

5'-GCGAATTCGATCCIACITT[TC]GC[GT]TTICC-3'.

5. A method according to claim 4 wherein the second primer includes the following sequence or part thereof:

5'-GCCTGCAGCCACATIC[TG][AG]TCIAC[AG]TT-3'.

6. A method according to claim 3 wherein the step of treating the RNA to construct cDNA includes

treating the RNA with reverse transcriptase and an adapter primer including the following sequence or part thereof:

5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3'

to form cDNA.

7. A method according to claim 2, which method further includes providing

a source of polypeptide having PPO activity,

a primer in sense orientation, and

an adapter primer;

isolating RNA from the source of polypeptide having PPO activity;

treating the RNA to construct cDNA therefrom; and

amplifying the cDNA so formed using the primers to prepare nucleic acid encoding the 3' end of PPO.

8. A method according to claim 7, which method further includes providing

a source of polypeptide having PPO activity,

an anchor,

primers in antisense orientation, and

an anchor primer;

isolating RNA from the source of polypeptide having PPO activity;

treating the RNA to construct cDNA therefrom;

attaching the anchor to the 5' end of the cDNA so formed; and

amplifying the cDNA using the primers to prepare nucleic acid encoding the 5' end of PPO.

9. A method according to claim 8 wherein the nucleic acid encodes lettuce PPO, the primer sense orientation includes the following sequence or part thereof:

5'-CGCTGGGTGGGTAATTCTAGGATG-3', and

the primers in antisense orientation include the following sequences or part thereof:

5'-TGCTGTTCTGTTCGAACATGGCAG-3'

5'-TATACAAGTGGCACCAGTGTCTGC-3'.

10. A method according to claim 8 wherein the nucleic acid encodes banana PPO, the primer in sense orientation includes the following sequence or part thereof:

5'-AGTCATCCACAATGCGGCGCACATG-3', and

the primers in antisense orientation include the following sequences or part thereof:

5'-CCGCATTGTGGATGACTTCCATCTG-3'

5'-CCAGAATGGGATGGTGAAGGTGTCG-3'.

11. A method according to claim 8 wherein the adapter primer includes the following sequence or part thereof:

5'-GACTCGAGTCGACATCG-3'.

12. A nucleic acid encoding banana PPO or antisense to banana PPO, fragments and derivatives thereof.

13. A nucleic acid according to claim 12 including a catalytic cleavage site.

14. A nucleic acid according to claim 12, having the sequence shown in Fig. 2 or Fig. 3, fragments and derivatives thereof, and substantially homologous sequences.

15. A nucleic acid encoding lettuce PPO or antisense to lettuce PPO, fragments and derivatives thereof.

16. A nucleic acid according to claim 15 including a catalytic cleavage site.

17. A nucleic acid according to claim 15, having the sequence shown in Fig. 1, fragments and derivatives thereof, and substantially homologous sequences.

18. A recombinant vector including a nucleic acid according to claim 12, which vector is capable of being replicated, transcribed and translated in a unicellular organism or alternatively in a plant.

19. A recombinant vector including a nucleic acid according to claim 15, which vector is capable of being replicated, transcribed and translated in a unicellular organism or alternatively in a plant.

20. A method of decreasing the level of PPO activity in a banana plant tissue, which method includes

providing

a nucleic acid according to claim 12; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic banana plant.

21. A method of decreasing the level of PPO activity in a lettuce plant tissue, which method includes

providing

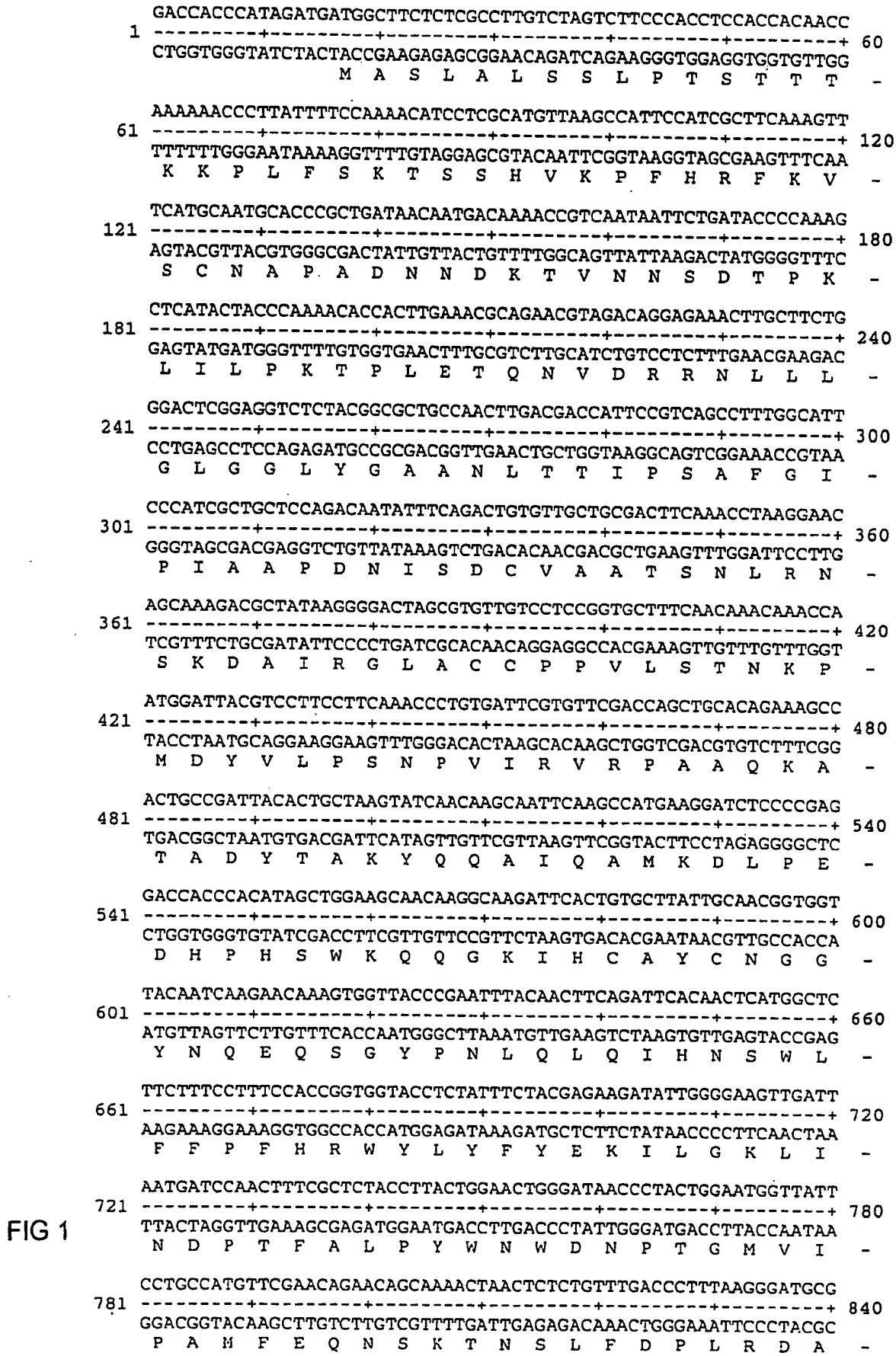
a nucleic acid according to claim 15; and

a plant sample; and

introducing said nucleic acid into said plant sample to produce a transgenic lettuce plant.

22. A transgenic banana plant, which plant contains nucleic acid capable of modifying expression of the normal banana PPO gene.

23. A transgenic lettuce plant, which plant contains nucleic acid capable of modifying expression of the normal lettuce PPO gene.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M A S L A L S S S L P T S T T T -
AAAAAACCCTTATTTTCCAAAACATCCTCGCATGTTAAGCCATTCCATCGCTTCAAAGTT
TTTTTTGGGAATAAAAGGTTTTGTAGGAGCGTACAATTCGGTAAGGTAGCGAAGTTTCAA
K K P L F S K T S S H V K P F H R F K V -
TCATGCAATGCACCCGCTGATAACAATGACAAAACCGTCAATAATTCTGATACCCCAAAG
AGTACGTTACGTGGGCGACTATTGTTACTGTTTTGGCAGTTATTAAGACTATGGGGTTTC
S C N A P A D N N D K T V N N S D T P K -
CTCATACTACCCAAAACACCACTTGAAACGCAGAACGTAGACAGGAGAAACTTGCTTCTG
GAGTATGATGGGTTTTGTGGTGAACTTTGCGTCTTGCATCTGTCCTCTTTGAACGAAGAC
L I L P K T P L E T Q N V D R R N L L L -
GGACTCGGAGGTCTCTACGGCGCTGCCAACTTGACGACCATTCCGTCAGCCTTTGGCATT
CCTGAGCCTCCAGAGATGCCGCGACGGTTGAACTGCTGGTAAGGCAGTCGGAAACCGTAA
G L G G L Y G A A N L T T I P S A F G I -
CCCATCGCTGCTCCAGACAATATTTCAGACTGTGTTGCTGCGACTTCAAACCTAAGGAAC
GGGTAGCGACGAGGTCTGTTATAAAGTCTGACACAACGACGCTGAAGTTTGGATTCCTTG
P I A A P D N I S D C V A A T S N L R N -
AGCAAAGACGCTATAAGGGGACTAGCGTGTTGTCCTCCGGTGCTTTCAACAAACAAACCA
TCGTTTCTGCGATATTCCCCTGATCGCACAACAGGAGGCCACGAAAGTTGTTTGTTTGGT
S K D A I R G L A C C P P V L S T N K P -
ATGGATTACGTCCTTCCTTCAAACCCTGTGATTCGTGTTCGACCAGCTGCACAGAAAGCC
TACCTAATGCAGGAAGGAAGTTTGGGACACTAAGCACAAGCTGGTCGACGTGTCTTTCGG
M D Y V L P S N P V I R V R P A A Q K A -
ACTGCCGATTACACTGCTAAGTATCAACAAGCAATTCAAGCCATGAAGGATCTCCCCGAG
TGACGGCTAATGTGACGATTCATAGTTGTTCGTTAAGTTCGGTACTTCCTAGAGGGGCTC
T A D Y T A K Y Q Q A I Q A M K D L P E -
GACCACCCACATAGCTGGAAGCAACAAGGCAAGATTCACTGTGCTTATTGCAACGGTGGT
CTGGTGGGTGTATCGACCTTCGTTGTTCCGTTCTAAGTGACACGAATAACGTTGCCACCA
D H P H S W K Q Q G K I H C A Y C N G G -
TACAATCAAGAACAAAGTGGTTACCCGAATTTACAACTTCAGATTCACAACTCATGGCTC
ATGTTAGTTCTTGTTTCACCAATGGGCTTAAATGTTGAAGTCTAAGTGTTGAGTACCGAG
Y N Q E Q S G Y P N L Q L Q I H N S W L -
TTCTTTCCTTTCCACCGGTGGTACCTCTATTTCTACGAGAAGATATTGGGGAAGTTGATT
AAGAAAGGAAAGGTGGCCACCATGGAGATAAAGATGCTCTTCTATAACCCCTTCAACTAA
F F P F H R W Y L Y F Y E K I L G K L I -
AATGATCCAACTTTCGCTCTACCTTACTGGAACTGGGATAACCCTACTGGAATGGTTATT
TTACTAGGTTGAAAGCGAGATGGAATGACCTTGACCCTATTGGGATGACCTTACCAATAA
N D P T F A L P Y W N W D N P T G M V I -
CCTGCCATGTTCGAACAGAACAGCAAAACTAACTCTCTGTTTGACCCTTTAAGGGATGCG
GGACGGTACAAGCTTGTCTTGTCGTTTTGATTGAGAGACAAACTGGGAAATTCCCTACGC
P A M F E Q N S K T N S L F D P L R D A -

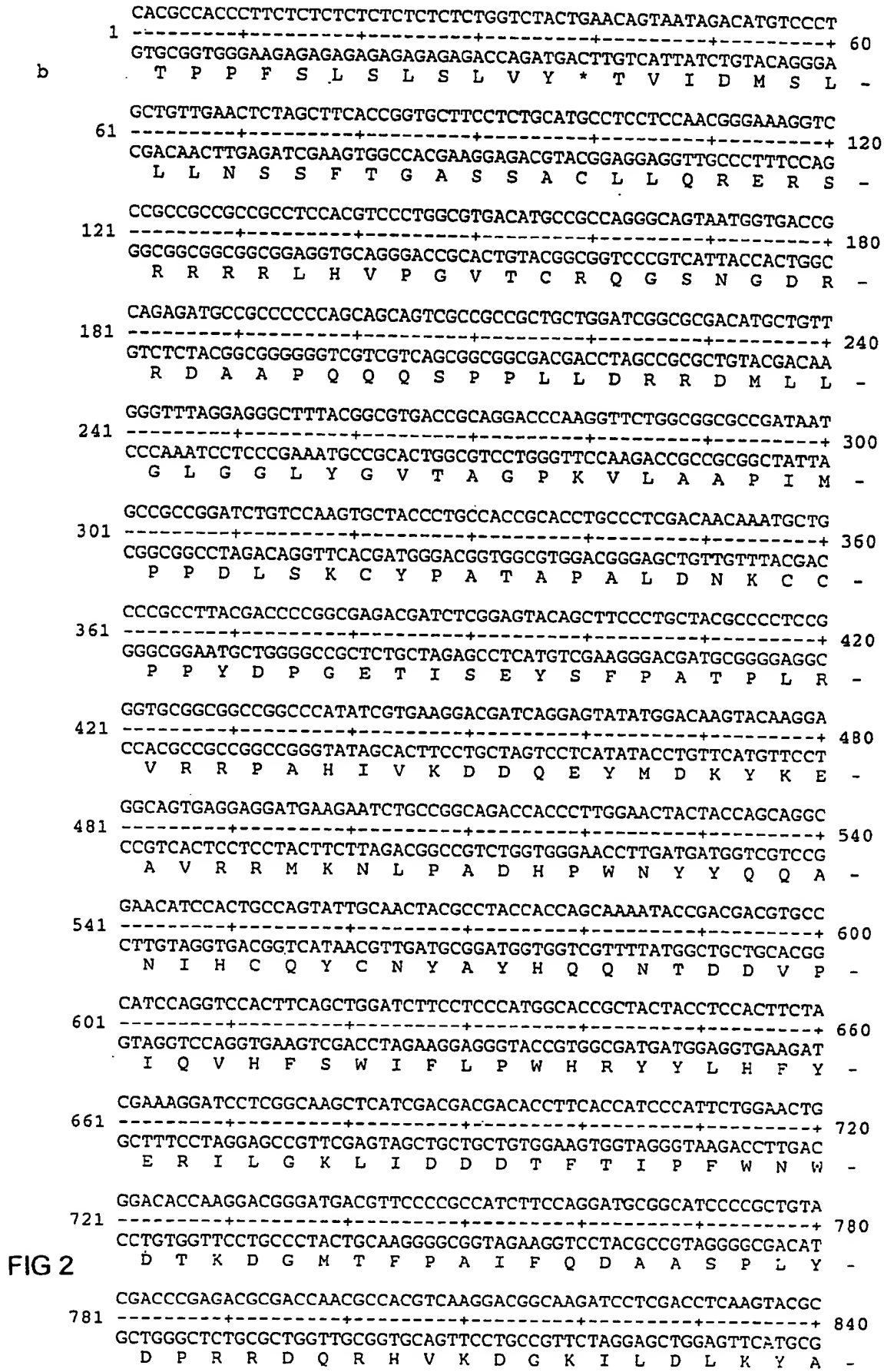
FIG 1

```
    AAACACCTCCCACCTTCTATCTTTGATGTTGAATATGCTGGTGCAGACACTGGTGCCACT
841 ---------+---------+---------+---------+---------+---------+ 900
    TTTGTGGAGGGTGGAAGATAGAAACTACAACTTATACGACCACGTCTGTGACCACGGTGA
     K  H  L  P  P  S  I  F  D  V  E  Y  A  G  A  D  T  G  A  T  -

    TGTATAGACCAGATAGCCATTAATCTGTCTTCAATGTACAGACAGATGGTCACCAACTCC
901 ---------+---------+---------+---------+---------+---------+ 960
    ACATATCTGGTCTATCGGTAATTAGACAGAAGTTACATGTCTGTCTACCAGTGGTTGAGG
     C  I  D  Q  I  A  I  N  L  S  S  M  Y  R  Q  M  V  T  N  S  -

    ACTGATACAAAACGATTCTTCGGTGGCGAATTTGTAGCTGGAAATGACCCTCTTGCGAGC
961 ---------+---------+---------+---------+---------+---------+ 1020
    TGACTATGTTTTGCTAAGAAGCCACCGCTTAAACATCGACCTTTACTGGGAGAACGCTCG
     T  D  T  K  R  F  F  G  G  E  F  V  A  G  N  D  P  L  A  S  -

    GAGTTCAACGTAGCTGGGACCGTAGAAGCTGGGGTTCACACTGCGGCTCACCGCTGGGTG
1021 ---------+---------+---------+---------+---------+---------+ 1080
    CTCAAGTTGCATCGACCCTGGCATCTTCGACCCCAAGTGTGACGCCGAGTGGCGACCCAC
     E  F  N  V  A  G  T  V  E  A  G  V  H  T  A  A  H  R  W  V  -

    GGTAATTCTAGGATGGCCAACAGCGAAGACATGGGGAACTTCTACTCCGCAGGATATGAT
1081 ---------+---------+---------+---------+---------+---------+ 1140
    CCATTAAGATCCTACCGGTTGTCGCTTCTGTACCCCTTGAAGATGAGGCGTCCTATACTA
     G  N  S  R  M  A  N  S  E  D  M  G  N  F  Y  S  A  G  Y  D  -

    CCTCTCTTTTACGTCCACCATGCGAATGTCGACAGGATGTGGCAAATCTGGAAAGATATT
1141 ---------+---------+---------+---------+---------+---------+ 1200
    GGAGAGAAAATGCAGGTGGTACGCTTACAGCTGTCCTACACCGTTTAGACCTTTCTATAA
     P  L  F  Y  V  H  H  A  N  V  D  R  M  W  Q  I  W  K  D  I  -

    GACAAGAAGACACACAAGGATCCGACCTCTGGCGACTGGCTAAATGCATCATACGTGTTT
1201 ---------+---------+---------+---------+---------+---------+ 1260
    CTGTTCTTCTGTGTGTTCCTAGGCTGGAGACCGCTGACCGATTTACGTAGTATGCACAAA
     D  K  K  T  H  K  D  P  T  S  G  D  W  L  N  A  S  Y  V  F  -

    TACGATGAGAATGAAAATCTTGTACGTGTCTACAACCGAGACTGTGTAGACATTAATCGG
1261 ---------+---------+---------+---------+---------+---------+ 1320
    ATGCTACTCTTACTTTTAGAACATGCACAGATGTTGGCTCTGACACATCTGTAATTAGCC
     Y  D  E  N  E  N  L  V  R  V  Y  N  R  D  C  V  D  I  N  R  -

    ATGGGATATGACTACGAAAGGTCAGCAATCCCATGGATCCGTAGTCGGCCGACTGCACAT
1321 ---------+---------+---------+---------+---------+---------+ 1380
    TACCCTATACTGATGCTTTCCAGTCGTTAGGGTACCTAGGCATCAGCCGGCTGACGTGTA
     M  G  Y  D  Y  E  R  S  A  I  P  W  I  R  S  R  P  T  A  H  -

    GCGAAGGGGGCGAACGTTGCTGCTAAGTCTGCTGGAATCGTGCAGAAGGTGGAGGATATC
1381 ---------+---------+---------+---------+---------+---------+ 1440
    CGCTTCCCCCGCTTGCAACGACGATTCAGACGACCTTAGCACGTCTTCCACCTCCTATAG
     A  K  G  A  N  V  A  A  K  S  A  G  I  V  Q  K  V  E  D  I  -

    GTATTCCCGCTGAAGTTAAACAAGATAGTGAAGGTTCTAGTGAAGAGGCCAGCTACAAAC
1441 ---------+---------+---------+---------+---------+---------+ 1500
    CATAAGGGCGACTTCAATTTGTTCTATCACTTCCAAGATCACTTCTCCGGTCGATGTTTG
     V  F  P  L  K  L  N  K  I  V  K  V  L  V  K  R  P  A  T  N  -

    AGGACCAAGGAGGGAAAGGAGAAAGCAAATGAGCTGTTGTTCGTGAATGGAATCACGTTT
1501 ---------+---------+---------+---------+---------+---------+ 1560
    TCCTGGTTCCTCCCTTTCCTCTTTCGTTTACTCGACAACAAGCACTTACCTTAGTGCAAA
     R  T  K  E  G  K  E  K  A  N  E  L  L  F  V  N  G  I  T  F  -

    GATGCTGAGCGGTTTCTAAAGATTGACGTGTTTGTCAACGACGTCGACGATGGAATTCAG
1561 ---------+---------+---------+---------+---------+---------+ 1620
    CTACGACTCGCCAAAGATTTCTAACTGCACAAACAGTTGCTGCAGCTGCTACCTTAAGTC
     D  A  E  R  F  L  K  I  D  V  F  V  N  D  V  D  D  G  I  Q  -

    ACCACCGCTGCTGATAGTGAGTTTGCTGGTAGTTTCGCACAGTTGCCACATAACCATGGC
1621 ---------+---------+---------+---------+---------+---------+ 1680
    TGGTGGCGACGACTATCACTCAAACGACCATCAAAGCGTGTCAACGTGTATTGGTACCCG
     T  T  A  A  D  S  E  F  A  G  S  F  A  Q  L  P  H  N  H  G  -
```

FIG 1 (cont.)

```
                GACAAGATGTTTATGAGGAGTGGGGCAGCGTTCGGGATCACGGAGCTCTTGGAAGACATT
1681            ---------+---------+---------+---------+---------+---------+ 1740
                CTGTTCTACAAATACTCCTCACCCCGTCGCAAGCCCTAGTGCCTCGAGAACCTTCTGTAA
                 D  K  M  F  M  R  S  G  A  A  F  G  I  T  E  L  L  E  D  I  -

                GAAGCTGAAGGTGATGACTCTGTTGTTGTGACATTGGTGCCGAGAACAGGGTGTGATGAA
1741            ---------+---------+---------+---------+---------+---------+ 1800
                CTTCGACTTCCACTACTGAGACAACAACACTGTAACCACGGCTCTTGTCCCACACTACTT
                 E  A  E  G  D  D  S  V  V  V  T  L  V  P  R  T  G  C  D  E  -

                GTAACTATTGGCGAGATCAAGATTCAGCTGGTTCCCATTGTTTAAAGTCTATTGAAGTAA
1801            ---------+---------+---------+---------+---------+---------+ 1860
                CATTGATAACCGCTCTAGTTCTAAGTCGACCAAGGGTAACAAATTTCAGATAACTTCATT
                 V  T  I  G  E  I  K  I  Q  L  V  P  I  V  *

                TGCATTTTCAATTGTCATTAGTATGCATGGGTACGTAAATCTGTTCGCTGTCTGGTTATC
1861            ---------+---------+---------+---------+---------+---------+ 1920
                ACGTAAAAGTTAACAGTAATCATACGTACCCATGCATTTAGACAAGCGACAGACCAATAG

                GAGGATTTTTGATGTTCTCGTAACCAAATAATAAGGATTGTCATTCCATGTTTGGAATCG
1921            ---------+---------+---------+---------+---------+---------+ 1980
                CTCCTAAAAACTACAAGAGCATTGGTTTATTATTCCTAACAGTAAGGTACAAACCTTAGC

                TGTAACCGCAGGCATGCATATGTTTGATTGTTATTTTTACTTGAAGCACTTCTGTTTTAG
1981            ---------+---------+---------+---------+---------+---------+ 2040
                ACATTGGCGTCCGTACGTATACAAACTAACAATAAAAATGAACTTCGTGAAGACAAAATC

                TAAAAAAAAAAAAAAAA
2041            ---------+------- 2057
                ATTTTTTTTTTTTTTTT
```

FIG 1
(cont.)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1
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b T P P F S L S L S L V Y * T V I D M S L -
GCTGTTGAACTCTAGCTTCACCGGTGCTTCCTCTGCATGCCTCCTCCAACGGGAAAGGTC
61
CGACAACTTGAGATCGAAGTGGCCACGAAGGAGACGTACGGAGGAGGTTGCCCTTTCCAG 120
L L N S S F T G A S S A C L L Q R E R S -
CCGCCGCCGCCGCCTCCACGTCCCTGGCGTGACATGCCGCCAGGGCAGTAATGGTGACCG
121
GGCGGCGGCGGCGGAGGTGCAGGGACCGCACTGTACGGCGGTCCCGTCATTACCACTGGC 180
R R R R L H V P G V T C R Q G S N G D R -
CAGAGATGCCGCCCCCCAGCAGCAGTCGCCGCCGCTGCTGGATCGGCGCGACATGCTGTT
181
GTCTCTACGGCGGGGGGTCGTCGTCAGCGGCGGCGACGACCTAGCCGCGCTGTACGACAA 240
R D A A P Q Q Q S P P L L D R R D M L L -
GGGTTTAGGAGGGCTTTACGGCGTGACCGCAGGACCCAAGGTTCTGGCGGCGCCGATAAT
241
CCCAAATCCTCCCGAAATGCCGCACTGGCGTCCTGGGTTCCAAGACCGCCGCGGCTATTA 300
G L G G L Y G V T A G P K V L A A P I M -
GCCGCCGGATCTGTCCAAGTGCTACCCTGCCACCGCACCTGCCCTCGACAACAAATGCTG
301
CGGCGGCCTAGACAGGTTCACGATGGGACGGTGGCGTGGACGGGAGCTGTTGTTTACGAC 360
P P D L S K C Y P A T A P A L D N K C C -
CCCGCCTTACGACCCCGGCGAGACGATCTCGGAGTACAGCTTCCCTGCTACGCCCCTCCG
361
GGGCGGAATGCTGGGGCCGCTCTGCTAGAGCCTCATGTCGAAGGGACGATGCGGGGAGGC 420
P P Y D P G E T I S E Y S F P A T P L R -
GGTGCGGCGGCCGGCCCATATCGTGAAGGACGATCAGGAGTATATGGACAAGTACAAGGA
421
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V R R P A H I V K D D Q E Y M D K Y K E -
GGCAGTGAGGAGGATGAAGAATCTGCCGGCAGACCACCCTTGGAACTACTACCAGCAGGC
481
CCGTCACTCCTCCTACTTCTTAGACGGCCGTCTGGTGGGAACCTTGATGATGGTCGTCCG 540
A V R R M K N L P A D H P W N Y Y Q Q A -
GAACATCCACTGCCAGTATTGCAACTACGCCTACCACCAGCAAAATACCGACGACGTGCC
541
CTTGTAGGTGACGGTCATAACGTTGATGCGGATGGTGGTCGTTTTATGGCTGCTGCACGG 600
N I H C Q Y C N Y A Y H Q Q N T D D V P -
CATCCAGGTCCACTTCAGCTGGATCTTCCTCCCATGGCACCGCTACTACCTCCACTTCTA
601
GTAGGTCCAGGTGAAGTCGACCTAGAAGGAGGGTACCGTGGCGATGATGGAGGTGAAGAT 660
I Q V H F S W I F L P W H R Y Y L H F Y -
CGAAAGGATCCTCGGCAAGCTCATCGACGACGACACCTTCACCATCCCATTCTGGAACTG
661
GCTTTCCTAGGAGCCGTTCGAGTAGCTGCTGCTGTGGAAGTGGTAGGGTAAGACCTTGAC 720
E R I L G K L I D D D T F T I P F W N W -
GGACACCAAGGACGGGATGACGTTCCCCGCCATCTTCCAGGATGCGGCATCCCCGCTGTA
721
CCTGTGGTTCCTGCCCTACTGCAAGGGGCGGTAGAAGGTCCTACGCCGTAGGGGCGACAT 780
D T K D G M T F P A I F Q D A A S P L Y -
CGACCCGAGACGCGACCAACGCCACGTCAAGGACGGCAAGATCCTCGACCTCAAGTACGC
781
GCTGGGCTCTGCGCTGGTTGCGGTGCAGTTCCTGCCGTTCTAGGAGCTGGAGTTCATGCG 840
D P R R D Q R H V K D G K I L D L K Y A -

FIG 2

```
                CTACACCGAAAACACTGCATCCGACAGCGAGATCATACGGGAGAACCTCTGCTTCATACA
841             ---------+---------+---------+---------+---------+---------+---------+ 900
                GATGTGGCTTTTGTGACGTAGGCTGTCGCTCTAGTATGCCCTCTTGGAGACGAAGTATGT
                  Y  T  E  N  T  A  S  D  S  E  I  I  R  E  N  L  C  F  I  Q  -

                GAAGACGTTCAAGCACAGCCTGTCGCTGGCGGAACTGTTCATGGGGGATCCCGTGCGCGC
901             ---------+---------+---------+---------+---------+---------+---------+ 960
                CTTCTGCAAGTTCGTGTCGGACAGCGACCGCCTTGACAAGTACCCCCTAGGGCACGCGCG
                  K  T  F  K  H  S  L  S  L  A  E  L  F  M  G  D  P  V  R  A  -

                GGGGGAGAAGGAGATCCAGGAGGCTAATGGGCAGATGGAAGTCATCCACAATGCGGCGCA
961             ---------+---------+---------+---------+---------+---------+---------+ 1020
                CCCCCTCTTCCTCTAGGTCCTCCGATTACCCGTCTACCTTCAGTAGGTGTTACGCCGCGT
                  G  E  K  E  I  Q  E  A  N  G  Q  M  E  V  I  H  N  A  A  H  -

                CATGTGGGTCGGAGAGCCGGACGGATACAAGGAAAACATGGGGGACTTCTCCACCGCCGC
1021            ---------+---------+---------+---------+---------+---------+---------+ 1080
                GTACACCCAGCCTCTCGGCCTGCCTATGTTCCTTTTGTACCCCCTGAAGAGGTGGCGGCG
                  M  W  V  G  E  P  D  G  Y  K  E  N  M  G  D  F  S  T  A  A  -

                CCGCGATTCTGTTTTCTTCTGCCACCATTCCAATGTCGACCGCATGTGGGACATCTACCG
1081            ---------+---------+---------+---------+---------+---------+---------+ 1140
                GGCGCTAAGACAAAAGAAGACGGTGGTAAGGTTACAGCTGGCGTACACCCTGTAGATGGC
                  R  D  S  V  F  F  C  H  H  S  N  V  D  R  M  W  D  I  Y  R  -

                CAACCTCCGCGGCAACCGCGTCGAGTTCGAAGACAACGACTGGTTGGACAGCACCTTCCT
1141            ---------+---------+---------+---------+---------+---------+---------+ 1200
                GTTGGAGGCGCCGTTGGCGCAGCTCAAGCTTCTGTTGCTGACCAACCTGTCGTGGAAGGA
                  N  L  R  G  N  R  V  E  F  E  D  N  D  W  L  D  S  T  F  L  -

                CTTCCACGACGAGAACGAACAGCTCGTCAAAGTCAAGATGAGCGACTGCCTCAACCCGAC
1201            ---------+---------+---------+---------+---------+---------+---------+ 1260
                GAAGGTGCTGCTCTTGCTTGTCGAGCAGTTTCAGTTCTACTCGCTGACGGAGTTGGGCTG
                  F  H  D  E  N  E  Q  L  V  K  V  K  M  S  D  C  L  N  P  T  -

                CAAGCTTCGGTACACGTTCGAGCAAGTTCCCCTCCCATGGCTGGGCAAAATCAATTGCCA
1261            ---------+---------+---------+---------+---------+---------+---------+ 1320
                GTTCGAAGCCATGTGCAAGCTCGTTCAAGGGGAGGGTACCGACCCGTTTTAGTTAACGGT
                  K  L  R  Y  T  F  E  Q  V  P  L  P  W  L  G  K  I  N  C  Q  -

                GAAGACGGCAGAGACGAAGTCCAAGGCCACGACGGAGCTGTCGCTGACGCGCGTGAACGA
1321            ---------+---------+---------+---------+---------+---------+---------+ 1380
                CTTCTGCCGTCTCTGCTTCAGGTTCCGGTGCTGCCTCGACAGCGACTGCGCGCACTTGCT
                  K  T  A  E  T  K  S  K  A  T  T  E  L  S  L  T  R  V  N  E  -

                ATTCGGGACGACGGCCCAGGCACTCGACGCGAGCAACCCGCTGCGGGTGATCGTGGCAAG
1381            ---------+---------+---------+---------+---------+---------+---------+ 1440
                TAAGCCCTGCTGCCGGGTCCGTGAGCTGCGCTCGTTGGGCGACGCCCACTAGCACCGTTC
                  F  G  T  T  A  Q  A  L  D  A  S  N  P  L  R  V  I  V  A  R  -

                GCCGAAGAAGAACCGCAAGAAGAAGGAGAAGCAAGAGAAGGTGGGGGTGATTCAGATCAA
1441            ---------+---------+---------+---------+---------+---------+---------+ 1500
                CGGCTTCTTCTTGGCGTTCTTCTTCCTCTTCGTTCTCTTCCACCCCCACTAAGTCTAGTT
                  P  K  K  N  R  K  K  K  E  K  Q  E  K  V  G  V  I  Q  I  K  -

                GGATATTAAGGTGACCACCAACGAGACAGCTCGCTTCGACGTCTATGTCGCGGTTCCTTA
1501            ---------+---------+---------+---------+---------+---------+---------+ 1560
                CCTATAATTCCACTGGTGGTTGCTCTGTCGAGCGAAGCTGCAGATACAGCGCCAAGGAAT
                  D  I  K  V  T  T  N  E  T  A  R  F  D  V  Y  V  A  V  P  Y  -

                CGGTGACCTCGCCGGACCCGACTACGGCGAGTTCGCGGGCAGCTACGTGAGGCTGGCGCA
1561            ---------+---------+---------+---------+---------+---------+---------+ 1620
                GCCACTGGAGCGGCCTGGGCTGATGCCGCTCAAGCGCCCGTCGATGCACTCCGACCGCGT
                  G  D  L  A  G  P  D  Y  G  E  F  A  G  S  Y  V  R  L  A  H  -

                TAGGATGAAGGGAAGCGACGGGACCGAAAAGCAGGGCCCCAAGAAGAAGGGAAAACTCAA
1621            ---------+---------+---------+---------+---------+---------+---------+ 1680
                ATCCTACTTCCCTTCGCTGCCCTGGCTTTTCGTCCCGGGGTTCTTCTTCCCTTTTGAGTT
                  R  M  K  G  S  D  G  T  E  K  Q  G  P  K  K  K  G  K  L  K  -
```

FIG 2
(cont.)

```
                GCTGGGTATTACGCCGCTGCTCGAGGACATCGATGCTGAGGACGCCGACAAGTTGGTGGT
1681            ---------+---------+---------+---------+---------+---------+
                CGACCCATAATGCGGCGACGAGCTCCTGTAGCTACGACTCCTGCGGCTGTTCAACCACCA
                 L  G  I  T  P  L  L  E  D  I  D  A  E  D  A  D  K  L  V  V  -

                CACCCTGGTTCTCCGCACTGGGAGCGTCACCGTGGGGGGAGTTTCCATCAATCTCCTGCA
1741            ---------+---------+---------+---------+---------+---------+
                GTGGGACCAAGAGGCGTGACCCTCGCAGTGGCACCCCCCTCAAAGGTAGTTAGAGGACGT
                  T  L  V  L  R  T  G  S  V  T  V  G  G  V  S  I  N  L  L  Q  -

                GACAGATTCTACCGCCGCCATCTAAATGATGGCCTCGGATCACAGCTTCTCCCCGCTTAA
1801            ---------+---------+---------+---------+---------+---------+
                CTGTCTAAGATGGCGGCGGTAGATTTACTACCGGAGCCTAGTGTCGAAGAGGGGCGAATT
                  T  D  S  T  A  A  I  *

                GTTGGAGTGATCGATTACTGGTGCTGCTTTCTTCCTCCCTGTCGTTCTTGCTATCTTCTT
1861            ---------+---------+---------+---------+---------+---------+
                CAACCTCACTAGCTAATGACCACGACGAAAGAAGGAGGGACAGCAAGAACGATAGAAGAA

                GATCTGGAACGATCCTTCAATAATTAGGGCATGACAGTAGTCGTCGCCCGATCCCATATG
1921            ---------+---------+---------+---------+---------+---------+
                CTAGACCTTGCTAGGAAGTTATTAATCCCGTACTGTCATCAGCAGCGGGCTAGGGTATAC

                TACGTGTTGGTCTCAACAGCTGTACATGTGACGTTATGGTGTGACTATATATTTTATTGC
1981            ---------+---------+---------+---------+---------+---------+
                ATGCACAACCAGAGTTGTCGACATGTACACTGCAATACCACACTGATATATAAAATAACG

                GGTCATCCTTGTTTCTTTCTTAAAAAAAAAAAAAAAAA
2041            ---------+---------+---------+-------- 2078
                CCAGTAGGAACAAAGAAAGAATTTTTTTTTTTTTTTTT
```

FIG 2
(cont.)

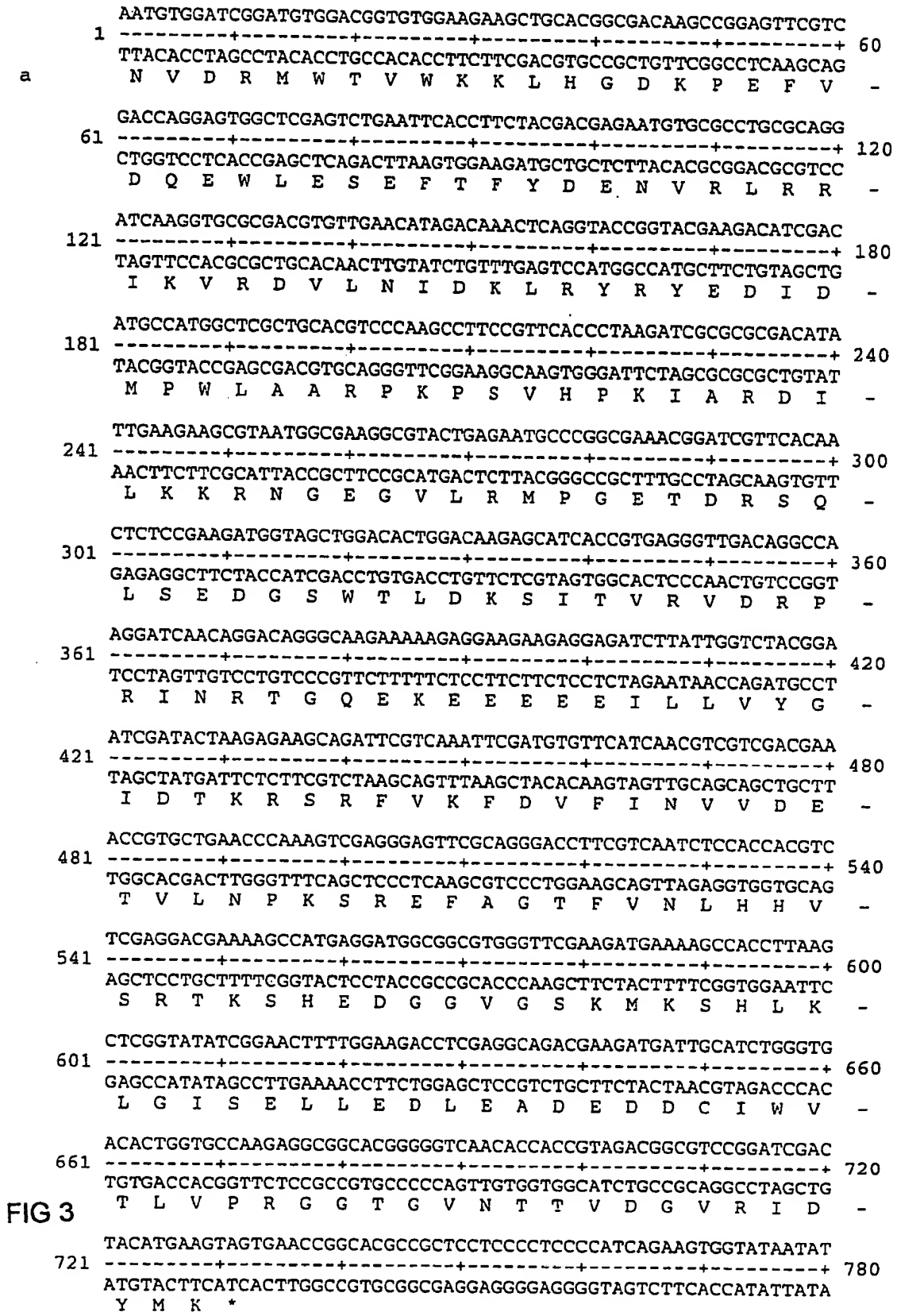
AATGTGGATCGGATGTGGACGGTGTGGAAGAAGCTGCACGGCGACAAGCCGGAGTTCGTC
1
60
TTACACCTAGCCTACACCTGCCACACCTTCTTCGACGTGCCGCTGTTCGGCCTCAAGCAG
a
N V D R M W T V W K K L H G D K P E F V -
GACCAGGAGTGGCTCGAGTCTGAATTCACCTTCTACGACGAGAATGTGCGCCTGCGCAGG
61
120
CTGGTCCTCACCGAGCTCAGACTTAAGTGGAAGATGCTGCTCTTACACGCGGACGCGTCC
D Q E W L E S E F T F Y D E N V R L R R -
ATCAAGGTGCGCGACGTGTTGAACATAGACAAACTCAGGTACCGGTACGAAGACATCGAC
121
180
TAGTTCCACGCGCTGCACAACTTGTATCTGTTTGAGTCCATGGCCATGCTTCTGTAGCTG
I K V R D V L N I D K L R Y R Y E D I D -
ATGCCATGGCTCGCTGCACGTCCCAAGCCTTCCGTTCACCCTAAGATCGCGCGCGACATA
181
240
TACGGTACCGAGCGACGTGCAGGGTTCGGAAGGCAAGTGGGATTCTAGCGCGCGCTGTAT
M P W L A A R P K P S V H P K I A R D I -
TTGAAGAAGCGTAATGGCGAAGGCGTACTGAGAATGCCCGGCGAAACGGATCGTTCACAA
241
300
AACTTCTTCGCATTACCGCTTCCGCATGACTCTTACGGGCCGCTTTGCCTAGCAAGTGTT
L K K R N G E G V L R M P G E T D R S Q -
CTCTCCGAAGATGGTAGCTGGACACTGGACAAGAGCATCACCGTGAGGGTTGACAGGCCA
301
360
GAGAGGCTTCTACCATCGACCTGTGACCTGTTCTCGTAGTGGCACTCCCAACTGTCCGGT
L S E D G S W T L D K S I T V R V D R P -
AGGATCAACAGGACAGGGCAAGAAAAAGAGGAAGAAGAGGAGATCTTATTGGTCTACGGA
361
420
TCCTAGTTGTCCTGTCCCGTTCTTTTTCTCCTTCTTCTCCTCTAGAATAACCAGATGCCT
R I N R T G Q E K E E E E E I L L V Y G -
ATCGATACTAAGAGAAGCAGATTCGTCAAATTCGATGTGTTCATCAACGTCGTCGACGAA
421
480
TAGCTATGATTCTCTTCGTCTAAGCAGTTTAAGCTACACAAGTAGTTGCAGCAGCTGCTT
I D T K R S R F V K F D V F I N V V D E -
ACCGTGCTGAACCCAAAGTCGAGGGAGTTCGCAGGGACCTTCGTCAATCTCCACCACGTC
481
540
TGGCACGACTTGGGTTTCAGCTCCCTCAAGCGTCCCTGGAAGCAGTTAGAGGTGGTGCAG
T V L N P K S R E F A G T F V N L H H V -
TCGAGGACGAAAAGCCATGAGGATGGCGGCGTGGGTTCGAAGATGAAAAGCCACCTTAAG
541
600
AGCTCCTGCTTTTCGGTACTCCTACCGCCGCACCCAAGCTTCTACTTTTCGGTGGAATTC
S R T K S H E D G G V G S K M K S H L K -
CTCGGTATATCGGAACTTTTGGAAGACCTCGAGGCAGACGAAGATGATTGCATCTGGGTG
601
660
GAGCCATATAGCCTTGAAAACCTTCTGGAGCTCCGTCTGCTTCTACTAACGTAGACCCAC
L G I S E L L E D L E A D E D D C I W V -
ACACTGGTGCCAAGAGGCGGCACGGGGGTCAACACCACCGTAGACGGCGTCCGGATCGAC
661
720
TGTGACCACGGTTCTCCGCCGTGCCCCCAGTTGTGGTGGCATCTGCCGCAGGCCTAGCTG
T L V P R G G T G V N T T V D G V R I D -
TACATGAAGTAGTGAACCGGCACGCCGCTCCTCCCCTCCCCATCAGAAGTGGTATAATAT
721
780
ATGTACTTCATCACTTGGCCGTGCGGCGAGGAGGGGAGGGGTAGTCTTCACCATATTATA
Y M K *

FIG 3

```
    TTATATTGGATCGAGGCTCGTGGTATCTTTTGATAAGAGTAAGTTCCATAAATTTAGAAG
781 ---------+---------+---------+---------+---------+---------+ 840
    AATATAACCTAGCTCCGAGCACCATAGAAAACTATTCTCATTCAAGGTATTTAAATCTTC

    AAGAATCATGTTCTTTATTTATATTAAATCAATGTGATTTGTCCAAAAAAAAAAAAAAAA
841 ---------+---------+---------+---------+---------+---------+ 900
    TTCTTAGTACAAGAAATAAATATAATTTAGTTACACTAAACAGGTTTTTTTTTTTTTTTT
```

FIG 3
(cont.)

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00310

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/53, 15/29, 5/04; A01H 5/00, 1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC6: C12N, A01H. Chemical Abstracts. All through Electronic Databases

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Biotechnology Abstracts Through Electronic Databases

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT DATABASES: WPAT & JAPIO Search terms: polyphenol()oxidase#, poly()phenol()oxidase#, ppo#, catechol()oxidase#, tryosinase#, diphenol()oxidase#, phenolase#, diphenolase#, mono()phenol()mono()oxygenase#, monophenol()mono()oxygenase#, brown:, C12N-015/IC, A01H/IC.
"BIOT" Search terms: EC-1.10.3.1 and A1/CL
"CASM" Search terms: The primers of claims 4, 5, 9, 10 and 11 were searched as partial nucleotide sequences. Also searched were polyphenol()oxidase#, diphenol()oxidase#, phenolase#, diphenolase#, gene#.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 93/2195 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 4 February 1993 Int Cl[5]: C12N 15/53, 9/02 See entire document	1-7 2,22,23
X	I.B.Dry & S.P. Robinson: "Molecular cloning and characterisation of grape berry polyphenol oxidase". Plant Molecular Biology, Vol 26, pp 495-502, 1994 See entire document	1,2

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 June 1996	02 JUL 1996
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer ROBYN PORTER Telephone No.: (06) 283 2318

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation f document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Derwent BIOT Online abstract Accession No. 90-12612. Abstracts of the Annual Meeting of the American Society for Microbiology, 90th Meeting, page 163, 1990. Williams et al: "Molecular cloning and partial characterisation of the polyphenol-oxidase (PPO) gene of Coriolus versicolor".	
X	See entire abstract	1
	WO 88/02372 (DONALD GUTHRIE FOUNDATION FOR MEDICAL RESEARCH), 7 April 1988, Int Cl[4]: C07H 15/12, C12Q 1/68, C12P 21/02, C12Q 1/26, C12P 13/22, C12N 15/00, 7/00.	
X		1
Y	See pages 1, 20-21, claims 1, 6, 7.	2
	Derwent BIOT Online Abstract Accession No. 95-05853. Abstracts of Papers of the American Chemical Society, 208th Meeting, Part 1, AGFD107, 1994. Steffans: "Modification of polyphenol-oxidase expression in crop plants".	
Y	See entire abstract.	20,21
	Derwent BIOT Online Abstract Accession No. 95-05846. Abstracts of Papers of the American Chemical Society, 208th Meeting, Part 1, AGFD2, 1994. Martinez and Whitaker: "Isolation of the gene encoding grape polyphenol-oxidase and study of PPO control with antisense RNA".	
Y	See entire abstract	20,21
	WO 94/03607 (KEYGENE N.V.) 17 February 1994, Int Cl[5]: C12N 15/53, 15/11, A01H 5/00.	
Y	See page 1 lines 3-11, claims 1, 15, 16 .	22,23
	WO 93/15599 (CORNELL RESEARCH FOUNDATION), 19 August 1993, Int Cl[5]: A01H 5/00, C12N 15/00, 15/29.	
Y	See claims 2, 3	22,23

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9302195	AU	23316/92	CA	2112998	EP	599868
		JP	7501686	NZ	243594		
WO	8802372	AU	81547/87	CA	1293940	EP	290504
		US	4898814				
WO	9403607	EP	606454	JP	7503376		
WO	9215599	EP	640613	JP	4275295	US	5470962

END OF ANNEX